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13. ABSTRACT (Maximum 200 Words)

We are developing a 'knock-in' mouse model with a mutation (glycine 525 to leucine, G525L) in estrogen receptor alpha (ERα) that permits exogenous regulation of its ligand-induced signaling pathways. This ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens but not to the synthetic nonsteroidal estrogen diethylstilbestrol (DES). Therefore, ERα signaling pathways can be 'turned on and off' in these mice through DES administration or withdrawal. To generate knock-in mice, an ERα construct containing the G525L mutation was engineered to facilitate homologous recombination into the mouse genome. The targeting construct was electroporated into ES cells, two positive clones were injected into mouse blastocysts, chimeras were generated, and germline transmission was established. Heterozygous mice were mated to produce litters of homozygous, heterozygous, and wild type mice. Genomic DNA from homozygous animals was sequenced and confirmed the G525L mutation was present. Reproductive tissues from 5 week old heterozygous and homozygous females were analyzed. Homozygous mice had immature and hypoplastic uterine tissue and mammary gland ductal trees. Homozygous ovaries were similar to those of heterozygous animals. Further analysis of this knock-in model will provide valuable information about the role of ERα in mammary gland development and carcinogenesis.

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INTRODUCTION

Estrogen receptor alpha (ERα) is a crucial therapeutic target for hormone dependent breast cancers. More effective treatment and prevention strategies are likely to emerge from an improved understanding of ERα mechanisms *in vivo*. To achieve this goal, we are developing a 'knock-in' mouse model with a mutation in ERα (glycine 525 to leucine, G525L) that permits exogenous regulation of its ligand-induced signaling pathways. This ligand-binding pocket mutation significantly reduces ERα response to endogenous estrogens but not to the synthetic nonsteroidal estrogen diethylstilbestrol (DES). Therefore, ERα signaling pathways can be 'turned on and off' in these mice through DES administration or withdrawal. These activities can be regulated both in developing animals as well as in adult animals exposed to tumorigenic agents, providing valuable information about the role of ERα in mammary gland development and carcinogenesis.

BODY

We previously anticipated that generation of ERα G525L mutant homozygous mice would be completed by the funding date. Although chimeric mice were generated, germline transmission was not established. Therefore, we repeated electroporation of the targeting construct into fresh embryonic stem (ES) cells. The 11.5 kilobase ERα construct, which was engineered to facilitate homologous recombination into the mouse genome, contained the G525L mutation (purple bar in green exon 9 box), an 18 bp 6xHis-tag epitope, and an extra Xba1 site (Figure 1). The original construct was obtained from Dr. Kenneth Korach[1] and the G525L mutation was inserted in our laboratory by site directed mutagenesis. We also inserted an ACN cassette into the targeting construct, obtained from Dr. Wondisford at the University of Chicago, which contained a testis-specific promoter (tACE), Cre structural gene, mouse RNA polymerase gene, and neo cassette, flanked at the 5' and 3' ends by loxP sites[2].

After electroporating the targeting construct into fresh ES cells, positive clones were identified by 5' external probe Southern blots (Figure 2) and 3' external primer polymerase chain reaction (PCR) analysis (Figure 3). Correct targeting was confirmed by PCR analysis of exon 9 and subsequent detection of the 6xHis-tag, Xba1 site (Figure 4), and G525L mutation (Figure 5). Two positive clones were injected into mouse blastocysts and chimeras were generated. The tACE promoter mediates expression of the Cre-recombinase gene in the testis of chimeric mice during spermatogenesis, thereby eliminating the ACN cassette in the ES cell derived sperm of the chimeras. The chimeras were mated with wild type mice to establish germline transmission. Heterozygous mice were mated to establish the F2 generation of wild type, heterozygous, and homozygous mice. Southern blot (Figure 6) and PCR strategies (Figure 7) were developed to genotype the animals and confirm removal of the ACN cassette in the F1 generation. ERα G525L mutant homozygous mice were obtained in January of 2005.

Task 1: To define the contribution of classical ERα activation in murine mammary gland development.

1. Analyze the G525L ERα knock-in mouse phenotype

a. We are currently developing a reverse transcriptase PCR (RT-PCR) strategy to confirm transgene expression. RT-PCR can be used to quantify mRNA levels from much smaller samples than an RNase protection assay and should provide better results. Our initial attempts to use immunohistochemistry (IHC) to detect the mutant ERα 6xHis-tag were unsuccessful in mouse uterine sections. We will continue to optimize our conditions for IHC and also try Western blots with extractions of protein from reproductive tissues with an antibody against the 6xHis-tag. Sequencing of genomic DNA confirmed the G525L mutation was present in homozygous animals.

b. To evaluate the phenotype of all potential sites of mutant ERα expression, reproductive tissues from 5 week old female mice were analyzed. IHC for ERα was performed on tissue sections from homozygous and heterozygous mutant ERα mice. Homozygous mice had immature and hypoplastic uterine tissue and a lack of estrogenization of the luminal and glandular epithelium (Figure 8). Homozygous ovaries were similar to those of heterozygous animals (Figure 9). However, these sections still need to be analyzed by a pathologist. In the future, the morphology of uterine tissue and ovaries, along with non-reproductive tissues like the bone, brain, and heart, in 3, 5, and 10 week old animals will be studied. We did not have enough wild-type mice to compare our results with in this preliminary experiment, but will use them in future experiments. We're also working on evaluating serum hormone concentrations and fertility.

c. Mammary gland whole mounts of 5 week old animals showed homozygous females have a rudimentary underdeveloped epithelial ductal tree, while heterozygous females have a ductal tree extending to the lymph node and enlarged terminal end buds (Figure 10). This indicates the homozygous mammary glands are unresponsive to estrogen. IHC for mammary gland sections showed similar ERα expression levels in the homozygous and heterozygous ducts (Figure 11). In the future, mammary gland development will be analyzed in mutant ERα homozygous, heterozygous, and wild-type mice at 3 and 10 weeks of age. mRNA and protein expression levels for ERα and PR will also be quantitated.

The remaining parts of task one and two will be completed in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Homozygous ERα G525L mutant mice were generated.
- Preliminary phenotypic analysis of reproductive tissues in homozygous and heterozygous females was performed in 5 week old mice.

REPORTABLE OUTCOMES

Animal Model Generation:
ERα G525L knock-in mice

Abstract:
Kerstin W. Sinkevicius, Karla A. Temple, Sonia L. Sugg, Fredric E. Wondisford, Kenneth S. Korach and Geoffrey L. Greene. Estrogen receptor alpha G525L knock-in

mice. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, 2005.

CONCLUSIONS

We have successfully generated 'knock-in' mice that have a mutation in ERα that permits exogenous regulation of its ligand-induced signaling pathways. Initial phenotypic analysis of 5 week old heterozygous and homozygous females indicated homozygous mice have immature and hypoplastic uterine tissue and mammary gland ductal trees but normal ovaries. Continued characterization of the model and DES treatment of the mutant ERα mice will increase our knowledge about ligand-induced, ligand-independent, and nongenomic signaling mechanisms of ERα *in vivo*. This information should facilitate the development of novel therapies for the treatment or prevention of breast cancer.

REFERENCES

1. Swope DL, Castranio T, Harrell JC, Mishina Y, Korach KS. AF-2 knock-in mutation of estrogen receptor alpha: cre-loxP excision of a PGK-neo cassette from the 3' UTR. Genesis. 2002;32:99-101.

2. Bunting M, Bernstein KE, Greer JM, Capecchi MR, Thomas KR. Targeting genes for self-excision in the germ line. Genes & Development. 1999;13:1524-8.

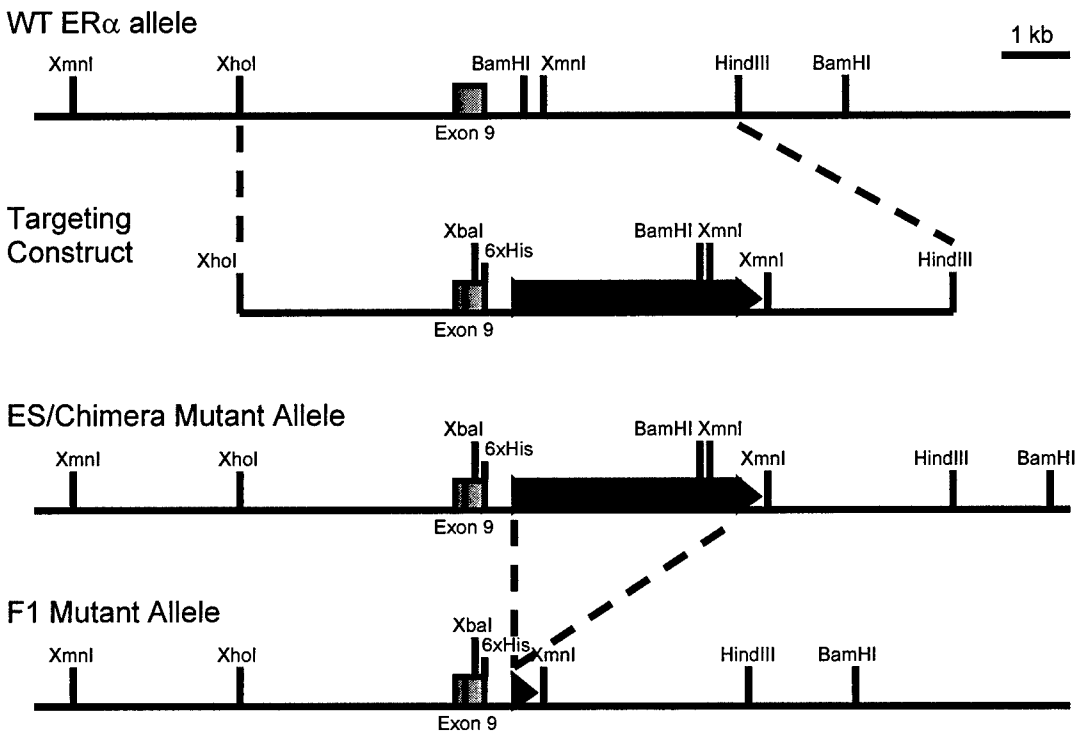

Figure 1. Targeting strategy for ERα G525L knock-in mutation.

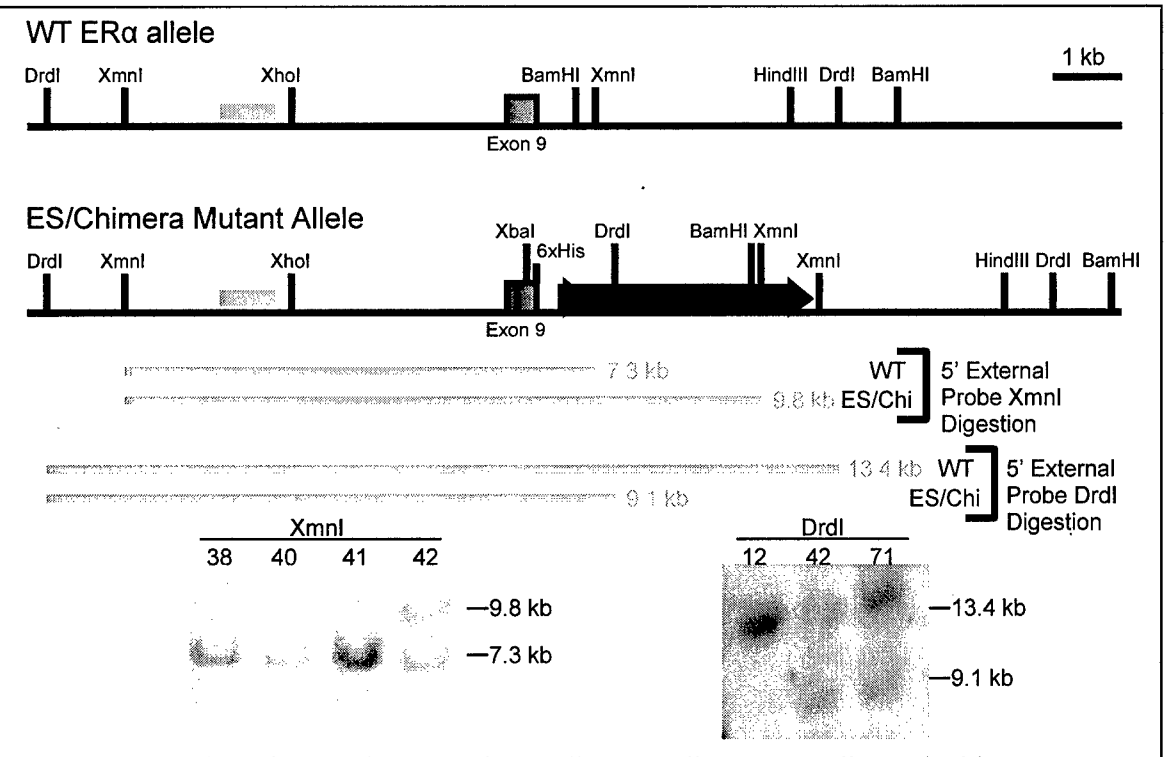

Figure 2. Detection of correctly targeted ES cells. ES cell DNA was digested with XmnI or DrdI and analyzed by Southern blotting using a 5' external probe (pink rectangle). Clones 42 and 71 are correctly inserted at the 5' end.

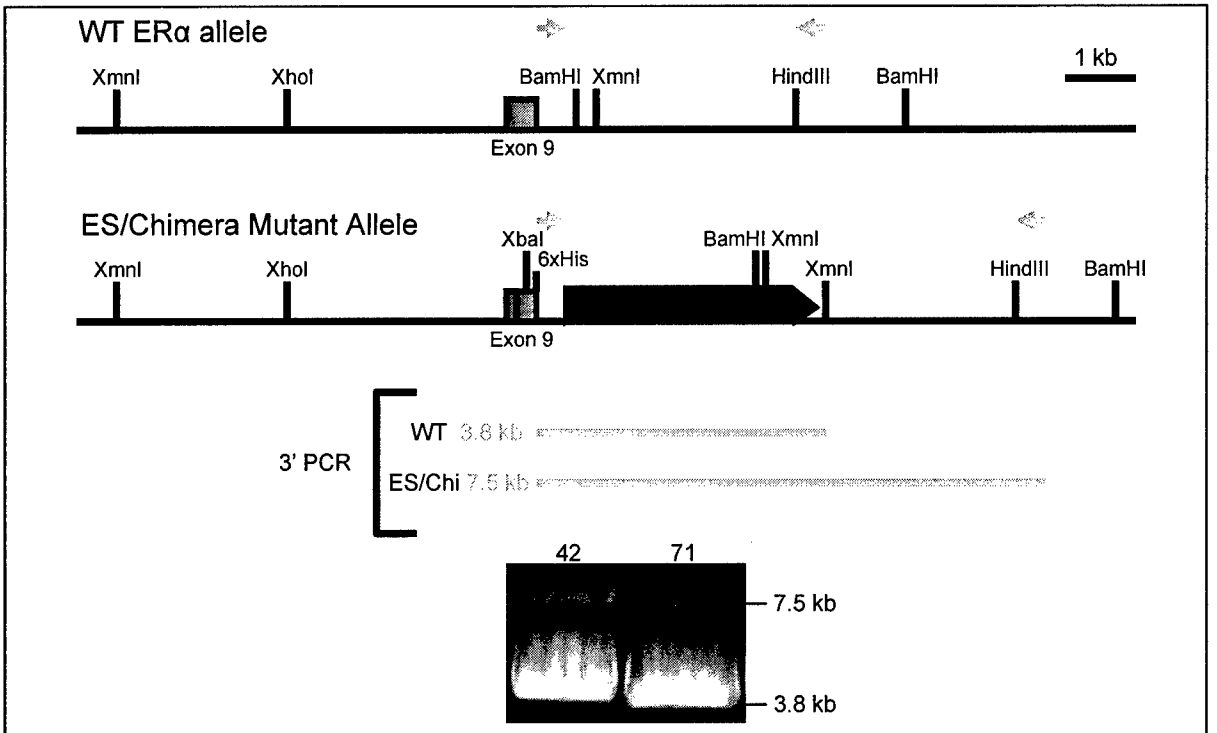

Figure 3. Detection of correctly targeted ES cell clones. ES cell DNA was analyzed by PCR using a 5' primer in the targeting construct and 3' external primer (pink arrows). Clones 42 and 71 are correctly inserted at the 3' end.

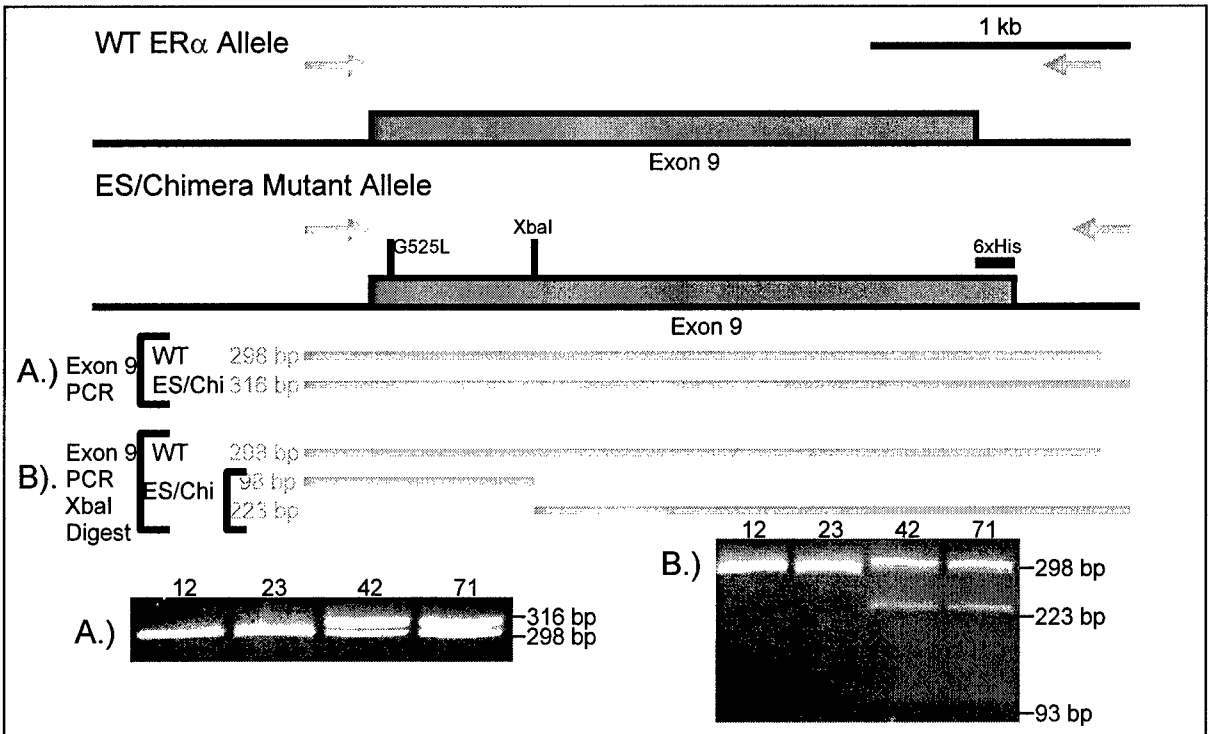

Figure 4. PCR analysis of the 6xHis-tag and XbaI site in exon 9. A.) ES cell DNA was analyzed by PCR using primers to exon 9 (pink arrows). The 6xHig-tag makes the targeted allele 18 bp longer than wild type. Clones 42 and 71 contain the 6xHis-tag. B.) XbaI digestion of the PCR product is only successful in correctly targeted clones 42 and 71.

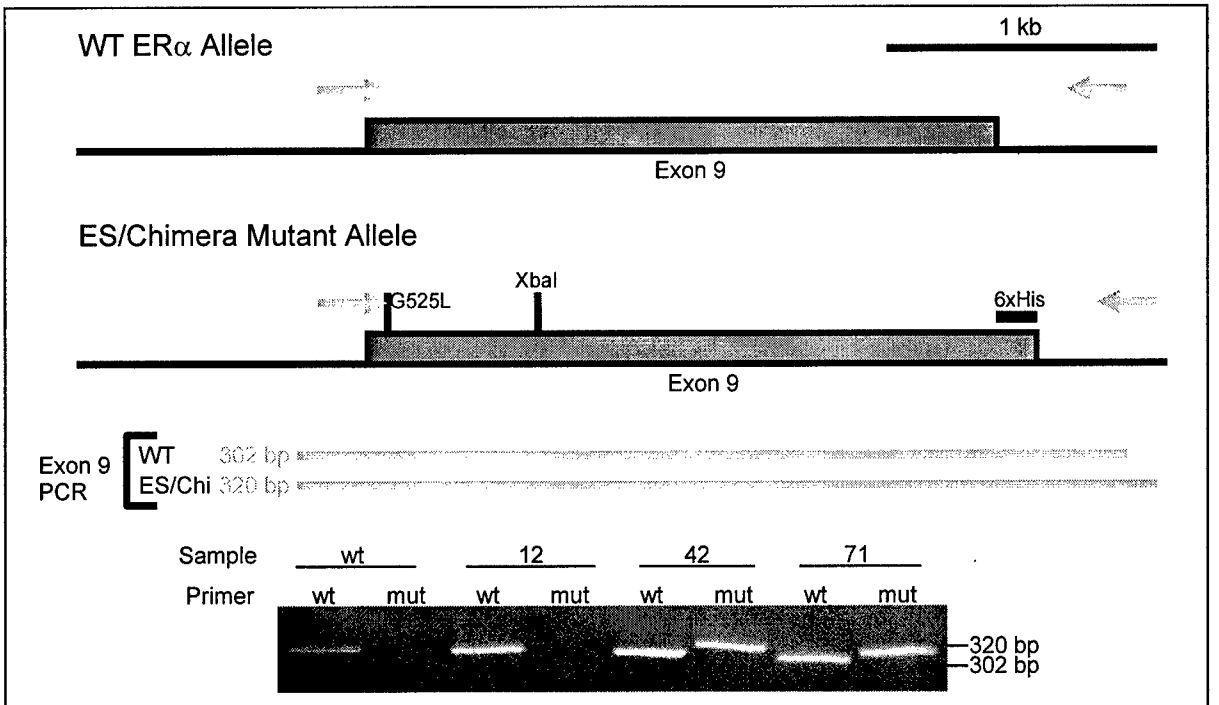

Figure 5. PCR analysis of the G525L mutation in exon 9. ES cell DNA was analyzed by PCR using 5' primers ending with wild type or mutant ERα sequence and a 3' primer (pink arrows). PCR product is amplified by both wild type (wt) and mutant (mut) 5' primers in correctly targeted clones 42 and 71.

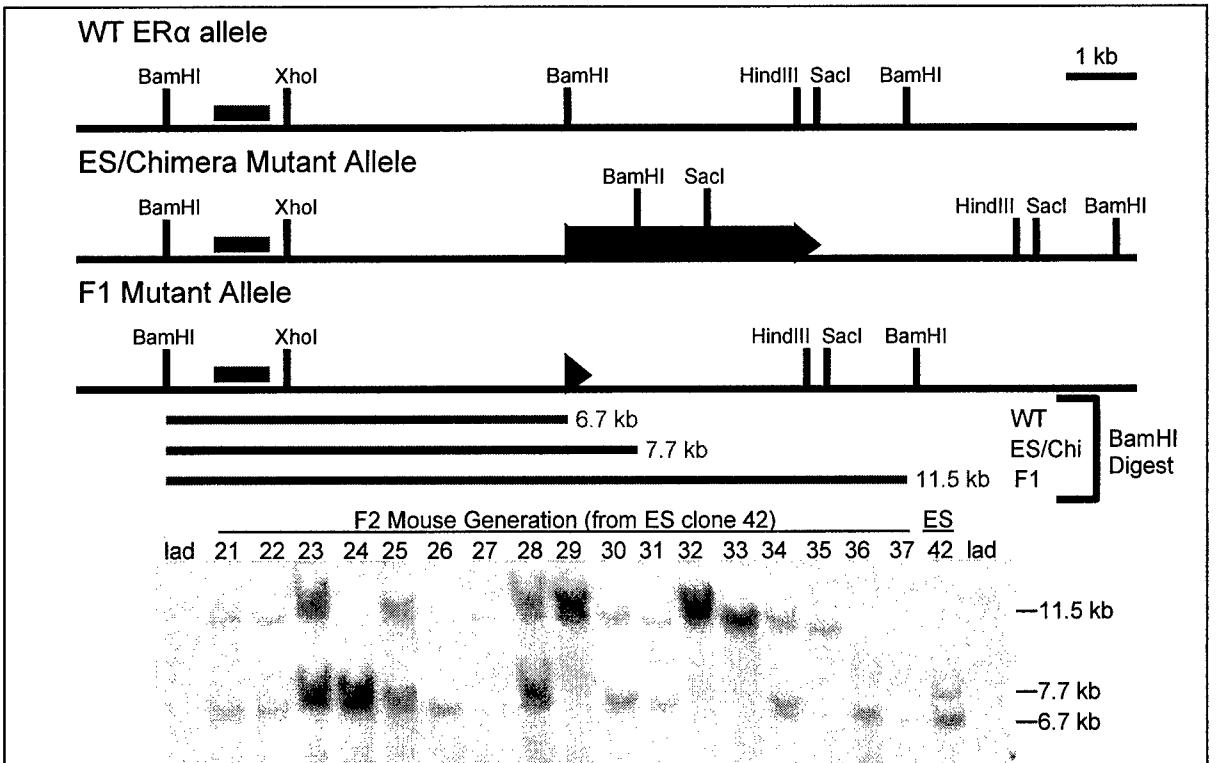

Figure 6. Southern blot genotyping strategy for F1 and F2 generations. DNA isolated from tail snips was digested with BamHI and analyzed by Southern blotting using a 5' external probe (red rectangle). F2 mice 21-37 are wild type, heterozygous, or homozygous. Clone 42 ES DNA and ladder (lad) were also run.

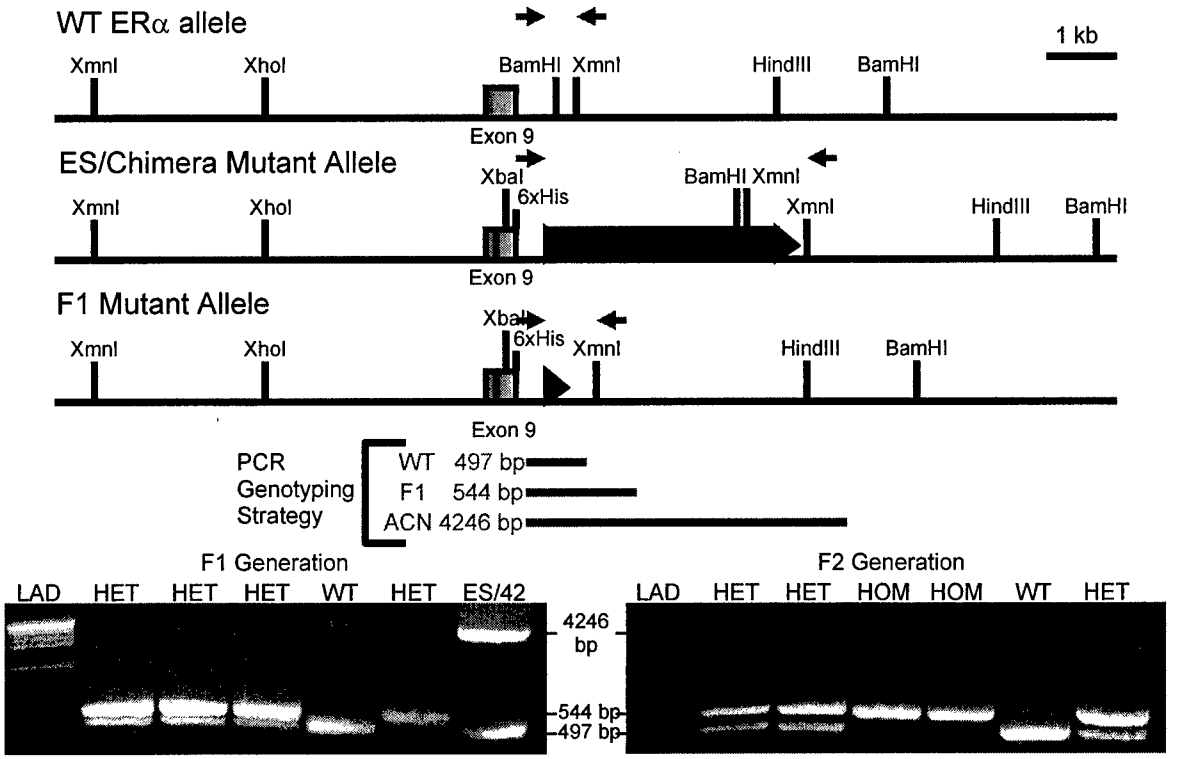

Figure 7. PCR genotyping strategy for F1 and F2 generations. DNA isolated from tail snips was analyzed by PCR using exon 9 primers (red arrows). F1 generation animals were wild type (wt) or heterozygous (het), confirming germline transmission. Ladder (lad) and clone 42 ES DNA were also run. F2 generation animals were wild type (wt), heterozygous (het), or homozygous (hom).

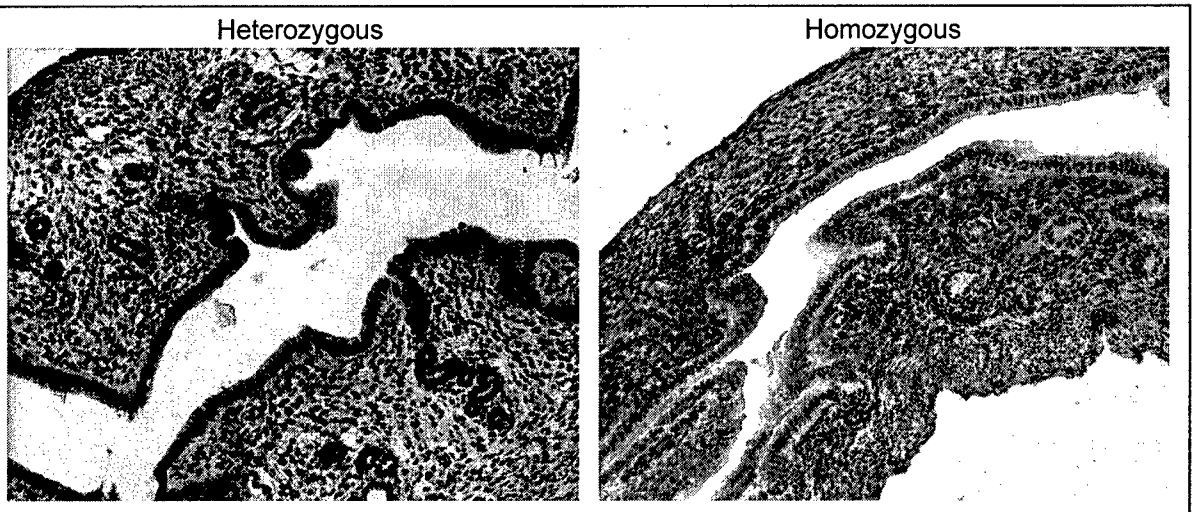

Figure 8. Uterine tissue immunohistochemical staining for ERα of representative 5 week old heterozygous and homozygous mutant ERα females. Homozygous cross sections depict the immature and hypoplastic uterine tissue and a lack of estrogenization of the luminal and glandular epithelium (20x). Note the smaller diameter of the homozygous uterus, as indicated by the ability to fit the whole transverse section of the tissue in the field of view.

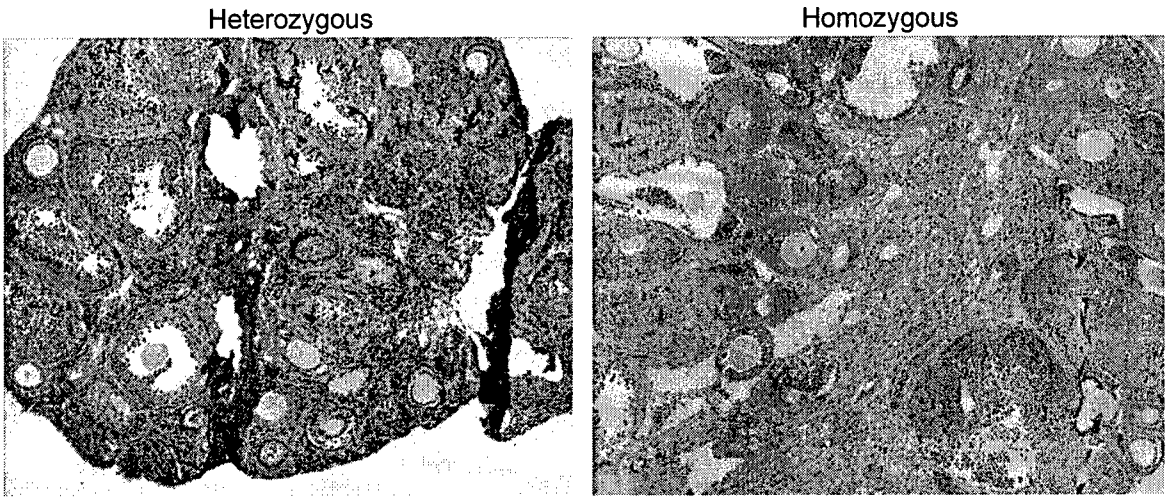

Figure 9. Ovarian tissue immunohistochemical staining for ERα of representative 5 week old heterozygous and homozygous mutant ERα females. Cross sections show no obvious differences between the heterozygous and homozygous ovaries (10x).

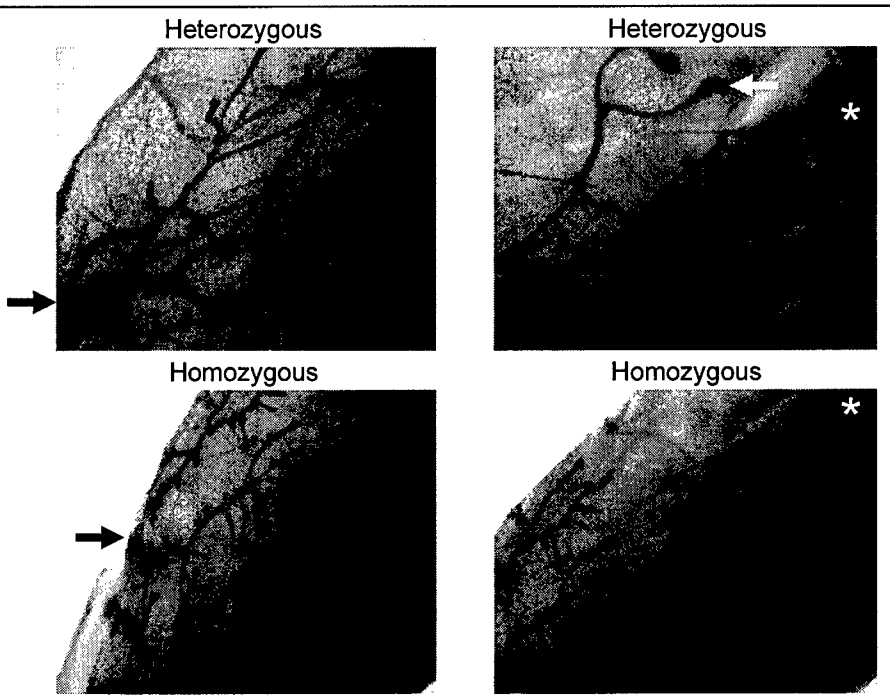

Figure 10. Mammary gland whole mounts of representative 5 week old heterozygous and homozygous mutant ERα females (4x). Homozygous females have a rudimentary underdeveloped epithelial ductal tree, while heterozygous females have a ductal tree extending to the lymph node (white asterisk) and enlarged terminal end buds (white arrow). The nipple is indicated by a black arrow.

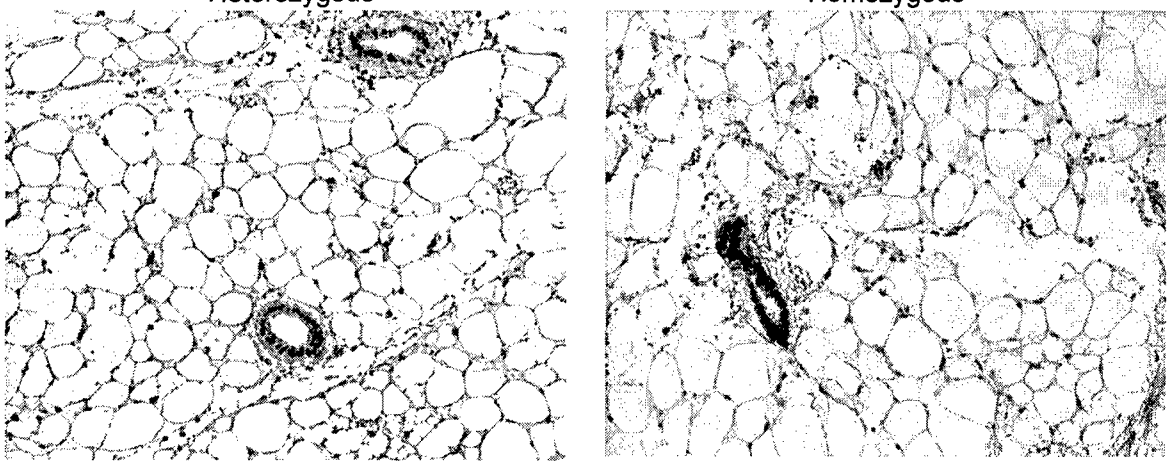

Figure 11. Mammary gland tissue immunohistochemical staining for ERα of representative 5 week old heterozygous and homozygous mutant ERα females. Mammary gland cross sections indicate similar ductal ERα expression levels in heterozygous and homozygous females (20x).